0026-895X/92/050753-07$03.00/0

MOLECULAR PHARMACOLOGY, 42:753-759

Coexisting β_1- and Atypical β-Adrenergic Receptors Cause Redundant Increases in Cyclic AMP in Human Neuroblastoma Cells

TIMOTHY A. ESBENSHADE, CHIDE HAN,[1] TRACEY L. THEROUX, JAMES G. GRANNEMAN, and KENNETH P. MINNEMAN

Department of Pharmacology, Emory University, Atlanta, Georgia 30322 (T.A.E., C.H., T.L.T., K.P.M.), and Cellular and Clinical Neurobiology Program, Department of Psychiatry, Wayne State University School of Medicine, Detroit, Michigan 48207 (J.G.G.)

Received May 21, 1992; Accepted August 10, 1992

SUMMARY

In SK-N-MC human neuroblastoma cells, the cAMP response to 10 nM isoproterenol (ISO) is mediated primarily by β_1-adrenergic receptors. However, responses to higher concentrations of ISO (100–1000 nM) were only weakly blocked by β_1- and β_2-selective antagonists. When β_1 receptors were blocked with 10 μM CGP 20712A, catecholamines still maximally activated cAMP accumulation, with only small decreases in potency. In the presence of CGP 20712A, β blockers inhibited the response to ISO stereoselectively but with relatively low potencies. Pindolol derivatives were partial agonists with low potencies, and the atypical agonist BRL 37344 was a partial agonist with an intermediate potency. All binding sites in these cells labeled by ^{125}I-cyanopindolol were of the β_1 subtype. Nuclease protection assays indicated that SK-N-MC cells contain mRNA for both the human β_1- and β_3-adrenergic receptors, with the β_3 subtype mRNA being expressed 25–50% more abundantly than that for the β_1 subtype. Northern blot hybridizations showed the presence of two β_3 mRNA transcripts of 3.1 and 2.4 kilobases. These results suggest that β_1- and atypical β-adrenergic receptors coexist in these cells and cause redundant increases in cAMP formation. Although molecular approaches suggest that the atypical subtype is the β_3, the observed drug specificity differs from that reported for the expressed recombinant human β_3 receptor.

It is now clear that each neurotransmitter exerts its effects through a large family of receptors. Nine adrenergic receptors for NE and EPI have been characterized and cloned (1–9), and the existence of additional receptors is likely (10–15). Adrenergic receptors are grouped into three families based upon structure, pharmacology, and signaling mechanisms (1). The α_1 family increases intracellular Ca^{2+} (2–4), the α_2 family inhibits adenylate cyclase (4–6), and the β family activates adenylate cyclase (7–9). Thus, simultaneous activation of multiple receptor subtypes can cause additive, opposing, synergistic, or independent effects (10), the biological implications of which have not been explored.

The human SK-N-MC human neuroblastoma cell line has been reported to contain exclusively the β_1 subtype (16), based on radioligand binding, cAMP accumulation, and Northern blots. However, in examining these cells we obtained unexpected results. We report here the coexistence of two β-adrenergic receptor subtypes in this cell line, i.e., the previously described β_1 subtype and a β-adrenergic receptor that pharmacologically resembles the "atypical" β subtype found in adipose tissue, heart, and intestine (12–15, 17–24) and that appears, by molecular approaches, to be the human β_3 subtype (9). The coexistence of these two subtypes, which cause redundant increases in cAMP, suggests that transmitters may simultaneously activate multiple receptor subtypes with unexpected pharmacological and functional consequences.

Experimental Procedures

Materials. SK-N-MC cells were obtained from the American Type Culture Collection (Rockville, MD). (−)- and (+)-Pindolol, (−)-hydroxybenzylpindolol, and (−)-CYP were from Sandoz (Basel, Switzerland). (−)- and (+)-Propranolol were from Ayerst (New York, NY). (+)-NE and (+)-EPI were from Sterling-Winthrop (Renssaeler, NY). Nadolol was kindly provided by Dr. Stephen Baker (University of Florida). (−)-ISO, (−)-NE, (−)-EPI, timolol, (−)-alprenolol, and all

This work was supported by National Science Foundation Grant DCB8908074 (K.P.M.) and National Institutes of Health Grants NS 21325 (K.P.M.) and DK 37006 (J.G.G.). T.A.E. is the recipient of a National Research Service Award (HL08448) from the National Institutes of Health, and C.H. is the recipient of a Biotechnology Career Fellowship from the Rockefeller Foundation.

[1] Permanent address: The Third Hospital, Beijing Medical University, Beijing 100083, China.

ABBREVIATIONS: NE, norepinephrine; ISO, isoproterenol; EPI, epinephrine; CGP, CGP 20712A; ICI, ICI 118,551; CYP, cyanopindolol; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Kb, kilobases; CHO, Chinese hamster ovary; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

other reagents were from Sigma (St. Louis, MO). [^{3}H]Adenine (20–40 Ci/mmol) and [α-^{32}P]ATP (800 Ci/mmol) were from DuPont NEN (Boston, MA). Carrier-free Na^{125}I was from Amersham (Arlington Heights, IL).

Cell culture. Cells were maintained in 90% Dulbecco's modified Eagle's medium with 10% fetal calf serum (GIBCO). Upon reaching confluence they were subcultured at a ratio of 1/5 to 1/10, in the same medium. Two milliliters were plated on 35-mm Primaria plates for cAMP determinations, for which cells were grown to confluency.

cAMP. cAMP accumulation was assessed by the [^{3}H]adenine prelabeling method (25), as described previously (26). Briefly, confluent 35-mm dishes were prelabeled with [^{3}H]adenine (1 μCi/2 ml) for 2 hr and washed twice with 1 ml of Krebs-Ringer bicarbonate buffer (composition, in mM: NaCl, 120; KCl, 5.5; $CaCl_2$, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $NaHCO_3$, 20; glucose, 11; $CaNa_2EGTA$, 0.029) at 37°. The buffer was aspirated and 1 ml of warm Krebs-Ringer bicarbonate buffer containing 0.2 mM IBMX was added. Drugs were then added and the plates were incubated for 10 min at 37°. The reactions were terminated by the addition of 0.1 ml of trichloroacetic acid, 50 μl of unlabeled cAMP were added as a carrier, and the plates were scraped. [^{3}H]cAMP was isolated by sequential Dowex and alumina chromatography (27). Data were calculated as a percentage of the conversion of [^{3}H]ATP to [^{3}H]cAMP.

Adenylate cyclase assay. Adenylate cyclase activity was assayed in cell homogenates prepared from SK-N-MC cells as described by Fishman *et al.* (16). In brief, 30–40 μg of cell homogenate protein were incubated in a final volume of 100 μl containing 25 mM Tris·HCl (pH 7.7), 1 mM dithiothreitol, 1 mM EDTA, 0.1% bovine serum albumin, 5 mM creatine phosphate, 5 unit of creatine phosphokinase, 1.5 mM $MgCl_2$, 200 μM IBMX, 1 mM cAMP, and 0.2 mM [α-^{32}P]ATP (1 μCi/tube). Reactions were incubated at 30° for 10 min, terminated, and analyzed for ^{32}P-labeled cAMP by sequential Dowex and alumina chromatography (27).

Radioligand binding. ^{125}I-CYP binding was measured in membrane preparations as described previously (16). Briefly, cells were washed twice in PBS (20 mM $NaPO_4$, 154 mM NaCl, pH 7.6), harvested, and homogenized in PBS. Homogenates were centrifuged at 30,000 × *g* for 10 min and pellets were resuspended in PBS (one confluent 100-mm plate/7 ml). Incubations were performed in a final volume of 0.25 ml of PBS containing 0.1 ml of tissue preparation and 50,000 cpm of ^{125}I-CYP (50 pM), in the presence or absence of competing drugs. Incubations were for 1 hr at 37° and were stopped by dilution with 10 ml of 10 mM Tris·HCl (pH 7.4) and filtration over glass fiber filters (Schleicher and Schuell no. 30). Filters were washed with an additional 10 ml of buffer and were counted in a γ-counter. Nonspecific binding was defined as binding remaining in the presence of 50 μM isoproterenol.

mRNA analysis. Probes for the human β_1- and β_3-adrenergic receptor mRNAs were obtained with the PCR. β_1 receptor cDNA was obtained by reverse transcription and PCR amplification as described previously (28), using oligonucleotide primers derived from the published human sequence (29). The resulting cDNA, encoding amino acids 178–271, was cloned into pGEM 7z (p145) and the sequence was verified. The human β_3 receptor probe was amplified from adipose tissue total nucleic acids by "nested" PCR (24), cloned into pGEM 7z, and sequenced. This probe (p146) encoded amino acids 151–223 of the human β_3-adrenergic receptor (9).

β_1 and β_3 receptor mRNAs were measured simultaneously in the same sample with a solution hybridization assay described previously (28). Briefly, radioactive cRNA probes were transcribed *in vitro* with [^{32}P]CTP, using the T7 promoter. The β_1 (p145) and the β_3 (p146) receptor cDNAs were linearized at the *Hind*III site in the vector. Cellular RNA (15–50 μg) was co-precipitated with 3×10^4 cpm of each ^{32}P-labeled probe. Samples were resuspended in 30 μl of hybridization buffer containing 75% formamide, 400 mM NaCl, 1 mM EDTA, and 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4, and were hybridized at 55° for 12–18 hr. Samples were diluted in 10 volumes of 300 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5, and 300 units of T-1 ribonuclease were added to each sample. Digestions were stopped after a 45-min incubation at 37°, and samples were precipitated in ethanol. Precipitates were resuspended with dye (bromphenol blue in 90% formamide, 10 mM EDTA, pH 7.5). The ^{32}P-labeled RNA probes that were protected from RNase digestion were electrophoretically resolved on a denaturing polyacrylamide gel containing 8 M urea. The gels were dried and exposed to Kodak XAR-5 film for 18–72 hr. The resulting autoradiograms were scanned with an E-C System densitometer coupled to a Shimadzu chromatograph integrator.

For quantitation of β receptor mRNAs, the ^{32}P-labeled cRNA probes were hybridized to known amounts (5–40 pg) of the corresponding nonradioactive sense RNA (transcribed *in vitro* from the SP-6 promoter) and processed as described above. The densitometric area of the autoradiographic bands was a linear function of the amount of sense RNA added. Quantitation of cellular β receptor mRNA levels was accomplished by comparing the autoradiographic signals of standards with those produced by tissue RNA.

Northern blot analysis was performed as previously described (24), using a randomly primed human β_3 probe derived from p146.

Results

Effects of β_1- and β_2-selective antagonists on responses to ISO and NE. Previous studies suggested that the human SK-N-MC neuroblastoma cell line contains exclusively β_1-adrenoceptors (16); however, we obtained unexpected results with these cells. Although the β_1-selective antagonist CGP potently inhibited the cAMP response to 10 nM ISO, as previously reported (K_i = 1 nM) (16), the potency of CGP was decreased >1000-fold when a 10-fold higher concentration of ISO was used (Fig. 1). Increasing the ISO concentration to 1 μM caused a further decrease in potency. Inhibition of the response to 50 nM NE by CGP was clearly biphasic, exhibiting both high and low affinity components (Fig. 1). The potency of CGP was reduced >200-fold when the NE concentration was increased 10-fold. The β_2-selective antagonist ICI showed a low potency in inhibiting responses to both agonists, with proportional decreases in potency occurring with increasing agonist concentration (Fig. 1).

Concentration-response curves for catecholamines. In order to further characterize this unusual dependency between the antagonism of cAMP accumulation by CGP and the concentration of agonist used, dose-response curves for the stimulation of cAMP accumulation by ISO, NE, and EPI were performed in control and CGP (10 μM)-treated cells. At 10 μM, CGP is present at a concentration that is 4 orders of magnitude greater than its K_i (2 nM) for the β_1-adrenergic receptor (see Fig. 5). Thus, a 5000-fold decrease in the potency of the agonists at the β_1-adrenergic receptor should be seen. In control cells, ISO, NE, and EPI increased cAMP accumulation with a rank order of potency suggestive of β_1-adrenoceptors (ISO > NE ≥ EPI) (Fig. 2). However, addition of 10 μM CGP to these cells caused only 5-, 23-, and 27-fold decreases in potency for NE, ISO, and EPI, respectively, instead of the 5000-fold shift expected if the agonists were acting solely at the β_1-adrenergic receptor. Each agonist produced nearly a full response in the presence of CGP, with ISO and NE being equally potent (Fig. 2). The α-adrenoceptor antagonist phentolamine (10 μM) did not affect agonist potency, either with or without CGP (data not shown). In addition, ISO stimulated adenylate cyclase activity in cell homogenates prepared from SK-N-MC cells in a concentration-dependent manner, with similar EC_{50} values in the absence and in the presence of CGP (1.0 and 3.1 μM,

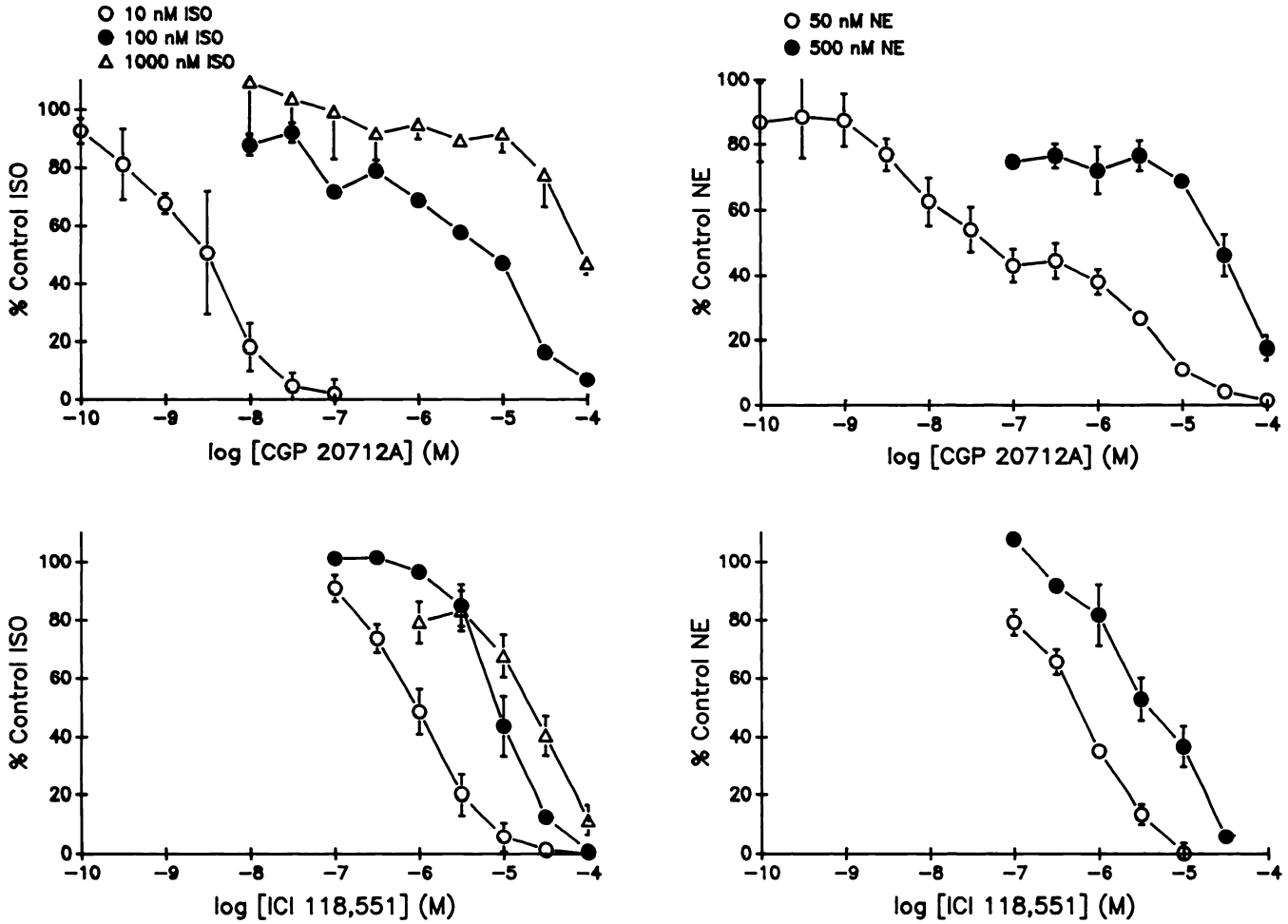

Fig. 1. Inhibition of cAMP accumulation by the β_1-selective antagonist CGP and the β_2-selective antagonist ICI with varying concentrations of agonists in SK-N-MC cells. Data are expressed as a percentage of the control response to the indicated concentrations of ISO or NE. ISO caused a maximal 19 ± 3-fold increase in cAMP accumulation. Each value is the mean ± standard error of data from two to four experiments performed in duplicate.

respectively) (Fig. 3). However, the maximal response to 100 μM ISO in the presence of 10 μM CGP was only 68% of that in the control cells. These findings suggest that two populations of β-adrenergic receptors exist in SK-N-MC cells. One population consists of the previously characterized β_1 receptor, which is potently activated by ISO, is quite sensitive to blockade by CGP, and exhibits an agonist order of potency of ISO > NE ≥ EPI. The other β receptor is activated by ISO with a lower potency and appears to be of neither the β_1 nor β_2 subtype, because it is not blocked by CGP and has a low affinity for the β_2 antagonist ICI. In addition, the rank order of potency of the agonists in the presence of CGP (ISO = NE > EPI) suggests the presence of an atypical β receptor in this cell line. Thus, these findings suggest that β_1-adrenergic receptors coexist with an atypical β-adrenergic receptor subtype in SK-N-MC cells and that either receptor alone can almost maximally increase cAMP.

Pharmacological characterization of the atypical subtype. In order to characterize these atypical β receptors in this cell line, we examined cAMP accumulation responses to various agonists and antagonists in the presence of 10 μM CGP. This concentration of CGP occupies 99.99% of the β_1-adrenergic receptors. We found that in the presence of 10 μM CGP activation by NE and EPI was stereoselective (Table 1). Additionally, the response to ISO (500 nM) was inhibited by a variety of β blockers with relatively low potencies. (−)-CYP was most potent, although much less potent than at β_1 or β_2 subtypes (7). (−)-Propranolol was about 100-fold more potent than (+)-propranolol, and betaxolol (β_1-selective) and ICI (β_2-selective) were very weak (Table 1). In the absence of CGP, pindolol derivatives were partial agonists, with (−)-hydroxybenzylpindolol having the highest potency and intrinsic activity (Fig. 4). (−)-Pindolol had a higher intrinsic activity than (+)-pindolol, and the atypical agonist BRL 37344 was a partial agonist with intermediate potency. The effects of (−)-hydroxybenzylpindolol (Fig. 4), (−)-pindolol, and BRL 37344 (data not shown) were not significantly antagonized by 10 μM CGP, providing further evidence that these compounds were stimulating cAMP accumulation via an atypical β-adrenergic receptor.

Radioligand binding. Binding sites labeled by ^{125}I-CYP were exclusively of the β_1 subtype (Fig. 5), as described previously (16). CGP, propranolol, nadolol, ICI (Fig. 5), and betaxolol (data not shown) all showed monophasic inhibition curves with Hill coefficients not significantly different from 1.0 (0.9–1.16). Attempts to label the atypical β-adrenergic receptors in either membranes or whole cells with ^{125}I-CYP were unsuccess-

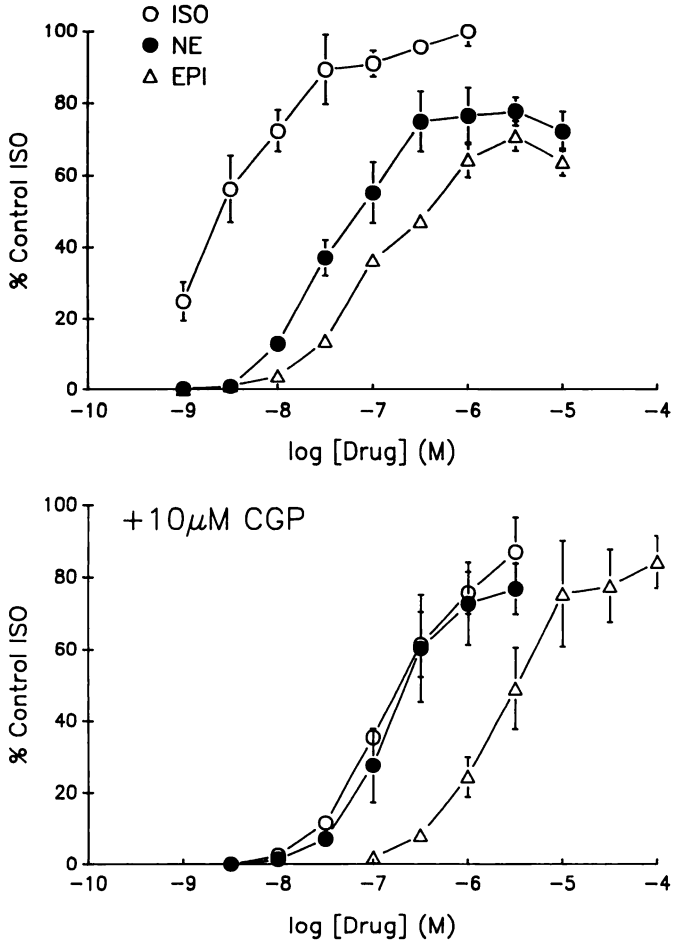

Fig. 2. Concentration-response curves for ISO-, NE-, and EPI-stimulated cAMP accumulation in SK-N-MC cells in the absence (*top*) and presence (*bottom*) of CGP (10 μM). cAMP accumulation was determined as described in Experimental Procedures. Data are expressed as a percentage of the response to 1 μM ISO in the absence of CGP. Each value is the mean ± standard error of data from three experiments performed in duplicate.

ful, even when high concentrations of ^{125}I-CYP were used (9). This is probably because of the very low affinity of CYP for this subtype (Table 1).

RNA analysis. The pharmacological properties of the atypical β-adrenergic receptor in this cell line are somewhat similar to those described for the cloned human β_3 receptor. However, a strict comparison between the pharmacology of this atypical β receptor and the expressed recombinant β_3 subtype is difficult because the information published on the cloned human β_3 receptor is scarce (9, 30). Therefore, we used molecular biological approaches in order to determine whether the SK-N-MC cell atypical β receptor is the β_3 receptor. The nuclease protection assay is a highly specific assay of gene expression, because only virtually perfect hybrids between cellular mRNA and the radioactive probes are protected from digestion by the nuclease. As predicted from previous work (16) as well as our studies, SK-N-MC cells contain mRNA encoding the β_1 receptor (Fig. 6A). Additionally, these cells clearly contain transcripts for the β_3 receptor. Indeed, β_3 receptor mRNA was about 25–50% more abundant than β_1 receptor mRNA in SK-N-MC cells. Northern blot analysis of total mRNA from these cells identified a predominant β_3 receptor mRNA species of 3.1 kb and a minor species of about 2.4 kb (Fig. 6B).

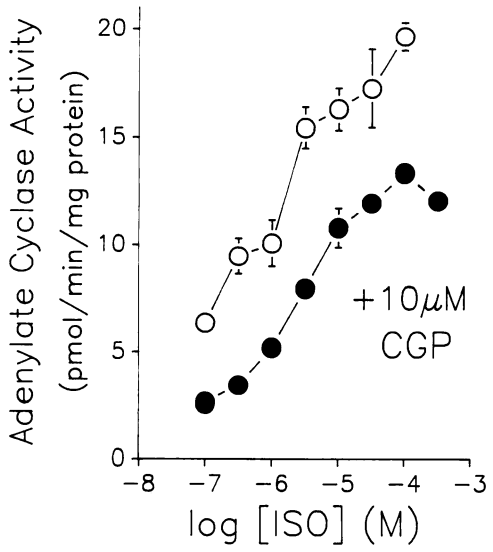

Fig. 3. Concentration-response curves for ISO-stimulated adenylate cyclase activity in cell homogenates prepared from SK-N-MC cells in the absence and presence of CGP (10 μM). Adenylate cyclase activity was determined as described in Experimental Procedures. Data are expressed as pmol of cAMP formed/min/mg of protein of cell homogenate. Each value is the mean ± standard error of two to four determinations of adenylate cyclase activity. Basal activity was 3.4 ± 0.02 pmol/min/mg of protein in control cells and 2.6 ± 0.2 pmol/min/mg of protein in CGP-treated cells.

TABLE 1

Pharmacological characterization of the atypical β-adrenoceptor in SK-N-MC cells

Concentration-response curves for each agonist were determined in the presence of 0.2 mM IBMX and 10 μM CGP in SK-N-MC cells, as described in Experimental Procedures. The EC_{50} value and percentage of the response to 1 μM ISO in the absence of CGP were calculated for each curve. Each value is the mean ± standard error of three experiments performed in duplicate. The potencies of the antagonists in blocking the cAMP response to ISO (500 nM) were determined in the presence of 10 μM CGP. IC_{50} values were calculated for each curve and converted to K_i values as described by Cheng and Prusoff (35). Each value is the mean ± standard error of three experiments performed in duplicate.

Agonists	EC_{50}	ISO maximum
	nM	% of control
(−)-ISO	152 ± 33	87 ± 9.8
(−)-NE	195 ± 58	77 ± 7.0
(−)-EPI	2,604 ± 958	84 ± 7.2
(+)-NE	12,200 ± 3,400	70 ± 8.2
(+)-EPI	41,800 ± 20,100	66 ± 2.7
(−)-Hydroxybenzylpindolol	190 ± 50	57 ± 3.3
(−)-CYP[a]	380 ± 140	15 ± 2.4
BRL 37344	840 ± 130	35 ± 1.6
(−)-Pindolol[a]	2,800 ± 1,080	36 ± 4.6
(+)-Pindolol	>20,000	3 ± 0.7
Antagonists	**K_i**	
	nM	
(−)-CYP[a]	28 ± 9	
(−)-Pindolol[a]	84 ± 12	
(−)-Propranolol	260 ± 47	
Timolol	260 ± 64	
(−)-Alprenolol	350 ± 21	
Nadolol	1,300 ± 280	
ICI	2,400 ± 560	
Betaxolol	19,400 ± 3,600	
(+)-Propranolol	20,300 ± 1,700	

[a] Mixed agonist/antagonist.

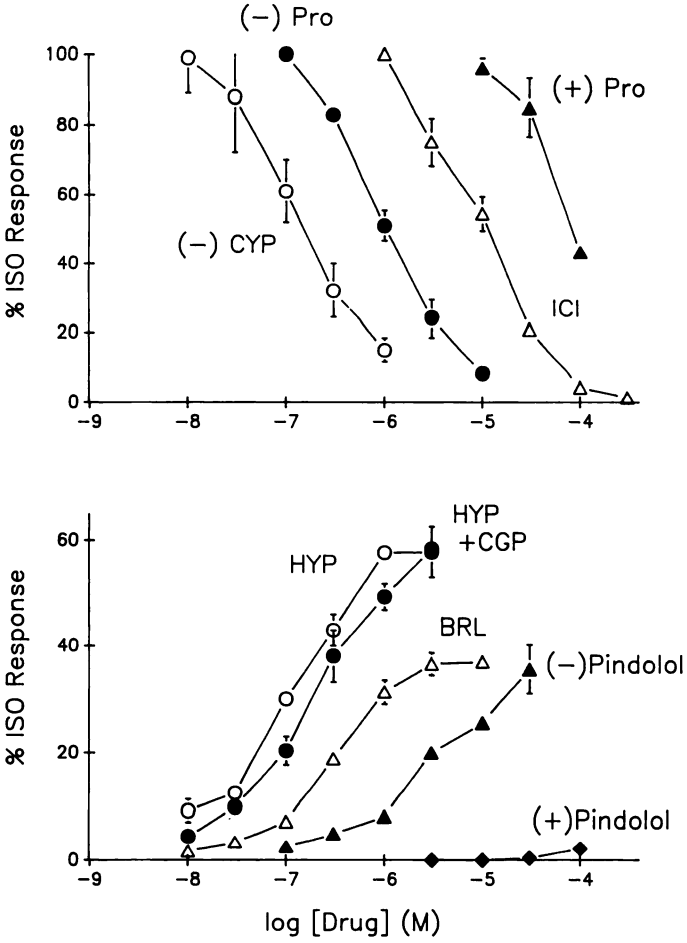

Fig. 4. Effect of antagonists and partial agonists on cAMP accumulation in SK-N-MC cells. *Top*, inhibition of responses to 500 nM ISO in the presence of 10 μM CGP by (−)-CYP, (−)- and (+)-propranolol [(−) and *(+)Pro*], and ICI. Data are expressed as a percentage of the response to 500 nM ISO in the presence of CGP. *Bottom*, increases in cAMP accumulation in SK-N-MC cells caused by (−)-hydroxybenzylpindolol (*HYP*) in the absence or presence (*+CGP*), of 10 μM CGP and by (−)- and (+)-pindolol and BRL 37344 (*BRL*) in the absence of CGP. Data are expressed as a percentage of the response to 1 μM ISO. Each value is the mean ± standard error of data from three experiments performed in duplicate.

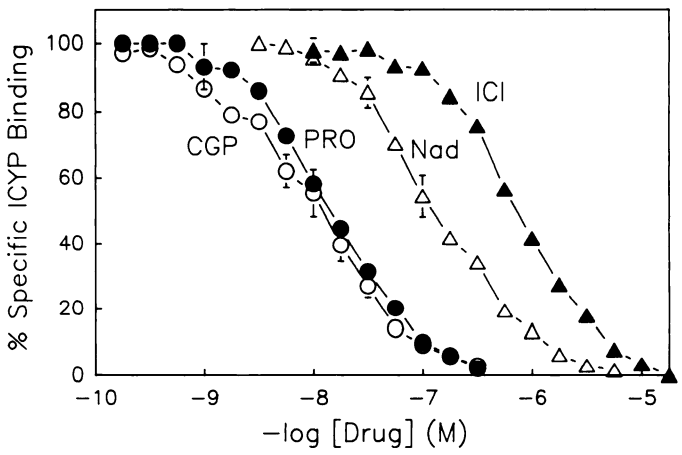

Fig. 5. Inhibition of specific ^{125}I-CYP binding by selective antagonists in membranes from SK-N-MC cells. Data are expressed as a percentage of specific binding in the absence of displacing drug. *PRO*, propranolol; *Nad*, nadolol. Each point is the mean ± standard error of data from three experiments performed in duplicate.

Discussion

These results suggest that two distinct β-adrenergic receptor subtypes coexist in this cell line and cause redundant increases in cAMP. One of these appears to be the β_1 subtype, as previously reported (16), and the other pharmacologically resembles the atypical β receptor described in a variety of mammalian tissues. In addition, these cells contain mRNA for both the β_1 and β_3 receptor subtypes.

The atypical β-adrenergic receptor in these cells has a rank order of agonist potency similar to that of the cloned human β_3 subtype (9), and pindolol derivatives and BRL 3744 are partial agonists at both. However, there are a number of differences in the drug specificities of the atypical β_3 receptor described here and the recombinant human β_3 receptor. The most dramatic difference is in the differential potency of typical β-adrenergic receptor antagonists. Propranolol and alprenolol inhibit this receptor with submicromolar potencies but have been reported to be essentially inactive at the β_3 subtype (9). It is difficult to compare the potencies of agonists because of possible differences in receptor reserves and problems in comparing data from binding and functional measurements. Based on the limited data available, however, it appears that CYP is about 100-fold less potent at this receptor than at the β_3 receptor (9). Pindolol has been reported to have EC_{50} values of 150 nM (9) or 1100 nM (30) in β_3-transfected CHO cells, whereas we found an EC_{50} of 2800 nM, although a K_i of 84 nM, in these cells. Similarly, the EC_{50} for BRL 37344 in β_3-transfected CHO cells has been reported to be 6 nM (9) and 180 nM (30) but is 840 nM in these cells. It appears that this subtype more closely resembles the atypical β-adrenergic receptor found in cardiac, intestinal, and adipose tissues (12–15, 18–22) and the cloned rat β_3 receptor (23, 24) than the cloned human β_3 receptor (9, 17, 30). Similarities include 1) a submicromolar affinity for classical β-adrenergic receptor antagonists, 2) a midnanomolar affinity for CYP, 3) activation by pindolol derivatives with low potency, and 4) a relatively low potency of BRL 37344.

Despite this pharmacological similarity to the atypical β-adrenergic receptor, highly specific nuclease protection assays suggest that the receptor that mediates the atypical β-adrenergic responses in the SK-N-MC cell line is the same as the cloned human β_3-adrenergic receptor. These cells were found to contain β_3 receptor transcripts in a relatively greater abundance than β_1 receptor transcripts. Two β_3 receptor mRNA transcripts, of 2.4 and 3.1 kb, were detected in Northern blot hybridizations using a human β_3 receptor cDNA probe.

Because human β_3 mRNA exists in these cells, it is not clear why we observed substantial differences between the pharmacological properties of this receptor and those published previously for the expressed recombinant human β_3-adrenergic receptor. Although the human β_3 receptor was cloned almost 3 years ago, little additional pharmacological characterization has been performed (9, 30). In contrast, the pharmacological evidence for the atypical β-adrenergic receptor in a variety of mammalian tissues is extensive and well defined (12–15, 18–22). In addition, the pharmacological profile of the expressed recombinant rat β_3 receptor more closely resembles that for the atypical β receptor than the cloned human β_3 receptor (23, 24). It is also possible that the properties of the β_3-adrenergic receptor natively expressed in SK-N-MC cells differ from those of the product of the cloned β_3 receptor cDNA transfected into

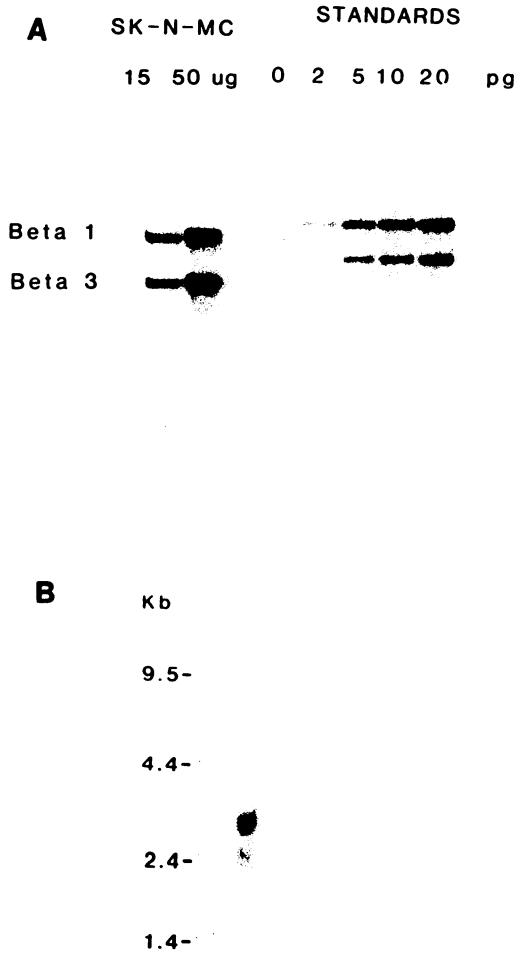

Fig. 6. β_1- and β_3-adrenergic receptor mRNA expression in SK-N-MC cells. A, RNase protection assay of total cellular RNA. Shown is an autoradiogram of ^{32}P-labeled β_1- and β_3 receptor cRNA probes that were protected from RNase digestion by cellular RNA or synthetic RNA standards. Probes that were protected by standards are larger than those protected by cellular RNA due to common vector sequences in the probes and standards. B, Northern blot analysis of SK-N-MC total RNA.

CHO cells. Indeed, the expression of the β_3 receptor in the SK-N-MC cell line may not be under normal regulatory controls, because these cells have numerous genetic abnormalities (e.g., polyploidy) and because the β_3 receptor is not expressed in the human brain.[2] The likelihood of this is reduced by the observation that transfection of β_1 and β_2 receptor cDNAs into a variety of cell types (including *Escherichia coli*) results in receptors with the expected pharmacological properties (31). Alternatively, this atypical β receptor may represent an additional atypical subtype, although it would have to be astonishingly homologous with the human β_3 receptor. Further study is necessary to clarify the reasons for these discrepancies.

These studies show that two closely related receptor subtypes coexist in these cells and activate redundant responses with important consequences. In intact cells, the response at low agonist concentrations is mediated primarily by the β_1 subtype, and this subtype alone can cause almost maximal increases in cAMP. The atypical subtype is activated at higher concentrations without observable effects. When both subtypes are activated in whole cells, the β_1-adrenergic receptor can be blocked without reduction in response. Similar redundancy has been reported for lipolysis and smooth muscle relaxation (12–15, 18–22), which are complicated by cell heterogeneity.

In cell homogenates, ISO is capable of stimulating adenylate cyclase when the β_1 receptor is blocked, indicating the presence of the atypical subtype in this preparation, as well as in whole cells. However, unlike the whole-cell system, there appears to be a β_1 subtype component of the adenylate cyclase response that is eliminated by CGP and that may be additive with the atypical β receptor response in cell homogenates. Further experiments are necessary in order to characterize further the contribution of each β receptor subtype to the adenylate cyclase response in cell homogenates.

The functional role of such redundant receptors is obscure. The atypical β-adrenergic receptor may play a "backup" role to ensure responses to agonists when dominant β_1-adrenergic receptors are lost. Indeed, acute agonist exposure desensitizes β_1- but not β_3-adrenergic receptors in isolated rat white adipocytes (32). Alternatively, the rank order of potency of endogenous agonists (β_1, NE = EPI; atypical, NE > EPI) may be altered by different contributions from the two subtypes. Because the atypical β-adrenergic receptor has never been found in isolation but is seen only when other β-adrenergic receptors are blocked, its effects may usually be masked by a more dominant typical β-adrenergic receptor. If so, it may be more widespread than previously thought.

Atypical β-adrenergic receptors have been suggested to be resistant to desensitization. The β_3 subtype has fewer consensus sequences for phosphorylation by cAMP-dependent protein kinase (33). However, Fishman *et al.* (16) showed that the cAMP response to ISO diminishes upon prolonged exposure to ISO in SK-N-MC cells, implying that this response does desensitize. We have found that both β_1- and β_3-mediated responses show similar desensitization in response to agonist exposure in these cells,[3] as has been reported in rat adipocytes (28). Because of the possibility of cross-talk between these two receptors, which activate the same signaling mechanism, it will be intriguing to compare the down-regulation of these two subtypes in this cell line.

Pharmacological analysis is seriously complicated by the coexistence of two subtypes causing redundant responses. The potencies of antagonists depend on agonist concentration and relative occupancy of each subtype. When both subtypes are activated, the drug specificity is be a hybrid of both subtypes. The potencies of agonists and antagonists reflect the subtype at which they have the highest and lowest affinities, respectively. Clearly, accurate characterization must include examination of multiple concentrations of agonists and antagonists (34).

These studies show that human SK-N-MC cells express high levels of both β_1- and atypical β-adrenergic receptors. Although the pharmacological properties of this atypical subtype differ in important ways from those of the expressed recombinant human β_3 receptor, highly specific nuclease protection assays suggest that these are the same subtypes. This is the first

[2] J. G. Granneman, unpublished observations.

[3] R. K. Bandlish, S. Dennison, T. A. Esbenshade, and K. P. Minneman, unpublished observations.

evidence for the β_3 subtype in cells of neuronal origin and the first description of a continuous cell line in which one can investigate the actions, interactions, and regulation of both the β_1- and β_3-adrenergic receptors. Finally, the coexistence of two subtypes causing redundant responses in the same cell has important implications that must be clarified as the number of known receptors for each transmitter continues to increase.

Acknowledgments

We thank Dr. David Clarke (Syntex), Dr. Stephen Baker (University of Florida), and Dr. T. J. Murphy (Emory University) for helpful discussions and Yu-Ping Zeng and Donald Hastings III for technical assistance.

References

1. Lefkowitz, R. J., and M. G. Caron. Adrenergic receptors: models for the study of receptors coupled to guanine nucleotide regulatory proteins. *J. Biol. Chem.* **263**:4993–4996 (1988).
2. Minneman, K. P. α_1-Adrenergic receptor subtypes, inositol phosphates and sources of cell calcium. *Pharmacol. Rev.* **40**:87–119 (1988).
3. Lomasney, J. W., S. Cotecchia, W. Lorenz, W.-Y. Leung, D. A. Schwinn, T. L. Yang-Feng, M. Brownstein, R. J. Lefkowitz, and M. G. Caron. Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor, the gene for which is located on human chromosome 5. *J. Biol. Chem.* **266**:6365–6369 (1991).
4. Harrison, J. K., W. R. Pearson, and K. R. Lynch. Molecular characterization of α_1- and α_2-adrenoceptors. *Trends Pharmacol. Sci.* **12**:62–67 (1991).
5. Bylund, D. B., C. Ray-Prenger, and T. J. Murphy. *Alpha*-2A and *alpha*-2B adrenergic receptor subtypes: antagonist binding in tissues and cell lines containing only one subtype. *J. Pharmacol. Exp. Ther.* **245**:600–607 (1988).
6. Lorenz, W., J. W. Lomasney, S. Collins, J. W. Regan, M. G. Caron, and R. J. Lefkowitz. Expression of three α_2-adrenergic receptor subtypes in rat tissues: implications for α_2 receptor classification. *Mol. Pharmacol.* **38**:599–603 (1990).
7. Minneman, K. P., R. N. Pittman, and P. B. Molinoff. β-Adrenergic receptor subtypes: properties, distribution and regulation. *Annu. Rev. Neurosci.* **4**:419–461 (1981).
8. Strader, C. D., M. R. Candelore, E. Rands, and R. A. F. Dixon. β-Adrenergic receptor subtype is an intrinsic property of the receptor gene product. *Mol. Pharmacol.* **32**:179–183 (1987).
9. Emorine, L. J., S. Marullo, M.-M. Briend-Sutren, G. Patey, K. Tate, C. Delavier-Klutchko, and A. D. Strosberg. Molecular characterization of the human β_3-adrenergic receptor. *Science (Washington D. C.)* **245**:1118–1121 (1989).
10. Minneman, K. P., and B. A. Atkinson. Interaction of subtype-selective antagonists with α_1-adrenergic receptor-mediated second messenger responses in rat brain. *Mol. Pharmacol.* **40**:523–530 (1991).
11. Simonneaux, V., M. Ebadi, and D. B. Bylund. Identification and characterization of α_{2D}-adrenergic receptors in bovine pineal gland. *Mol. Pharmacol.* **40**:235–241 (1991).
12. Arch, J. R. S., A. T. Ainsworth, M. A. Cawthorne, V. Piercy, M. V. Sennitt, V. E. Thody, C. Wilson, and S. Wilson. Atypical β-adrenoceptors on brown adipocytes as target for anti-obesity drugs. *Nature (Lond.)* **309**:163–165 (1984).
13. Bond, R. A., and D. E. Clarke. Agonist and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the *alpha*- and *beta*-subtypes. *Br. J. Pharmacol.* **95**:723–734 (1988).
14. Kaumann, A. J. Is there a third heart β-adrenoceptor? *Trends Pharmacol. Sci.* **10**:316–320 (1989).
15. Zaagsma, J., and S. R. Nahorski. Is the adipocyte β-adrenoceptor a prototype for the recently cloned atypical "β_3-adrenoceptor"? *Trends Pharmacol. Sci.* **11**:3–7 (1990).
16. Fishman, P. H., E. Nussbaum, and R. S. Duman. Characterization and regulation of β_1-adrenergic receptors in a human neuroepithelioma cell line. *J. Neurochem.* **56**:596–602 (1991).
17. Tate, K. M., M.-M. Briend-Sutren, L. J. Emorine, C. Delavier-Klutchko, S. Marullo, and A. D. Strosberg. Expression of three human β-adrenergic receptor subtypes in transfected Chinese hamster ovary cells. *Eur. J. Biochem.* **196**:357–361 (1991).
18. Walter, M., H. Lemoine, and A. J. Kaumann. Stimulant and blocking actions of optical isomers of pindolol on the sinoatrial node and trachea of guinea pig: role of β-adrenoceptor subtypes in the dissociation between blockade and stimulation. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **327**:159–175 (1984).
19. Bianchetti, A., and L. Manara. *In vitro* inhibition of intestinal motility by phenylethanolmainotetralines: evidence of atypical β-adrenoceptors in rat colon. *Br. J. Pharmacol.* **100**:831–839 (1990).
20. Blue, D. R., R. A. Bond, N. Adham, R. Delmendo, A. D. Michel, R. M. Eglen, R. L. Whiting, and D. E. Clarke. Antagonist characterization of atypical *beta* adrenoceptors in guinea pig ileum: blockade by alprenolol and dihydroalprenolol. *J. Pharmacol. Exp. Ther.* **252**:1034–1042 (1990).
21. Granneman, J. G. Norepinephrine and BRL 37344 stimulate adenylate cyclase via different receptors in rat brown adipose tissue. *J. Pharmacol. Exp. Ther.* **254**:508–513 (1990).
22. Langin, D., M. P. Portillo, J.-S. Saulnier, and M. Lafontan. Coexistence of three β-adrenoceptor subtypes in white fat cells of various mammalian species. *Eur. J. Pharmacol.* **199**:291–301 (1991).
23. Muzzin, P., J.-P. Revelli, F. Kuhne, J. D. Gocayne, W. R. McCombie, J. C. Venter, J.-P. Giacobino, and C. M. Fraser. An adipose tissue-specific β-adrenergic receptor: molecular cloning and down-regulation in obesity. *J. Biol. Chem.* **266**:24053–24058 (1991).
24. Granneman, J. G., K. N. Lahners, and A. Chaudhry. Molecular cloning and expression of the rat β_3-adrenergic receptor. *Mol. Pharmacol.* **40**:895–899 (1991).
25. Shimizu, H., C. R. Creveling, and J. W. Daly. A radioisotopic method for measuring the formation of adenosine 3′5′-monophosphate in incubated slices of brain. *J. Neurochem.* **16**:1609–1616 (1969).
26. Atkinson, B. A., and K. P. Minneman. Multiple adrenergic receptor subtypes controlling cyclic AMP formation: comparison of brain slices and primary neuronal and glial cultures. *J. Neurochem.* **56**:587–595 (1990).
27. Salomon, Y. C., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
28. Granneman, J. G., and K. N. Lahners. Differential adrenergic regulation of β_1- and β_3-adrenoceptor messenger ribonucleic acids in adipose tissues. *Endocrinology* **130**:109–114 (1991).
29. Frielle, T., S. Collins, K. W. Daniel, M. G. Caron, R. J. Lefkowitz, and B. K. Kobilka. Cloning of the cDNA for the human β_1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84**:7920–7924 (1987).
30. Feve, B., L. J. Emorine, F. Lasnier, N. Blin, B. Baude, C. Nahmias, A. D. Strosberg, and J. Pairault. Atypical β-adrenergic receptor in 3T3-F442A adipocytes: pharmacological and molecular relationship with the human β_3-adrenergic receptor. *J. Biol. Chem.* **266**:20329–20336 (1991).
31. Marullo, S., C. Delavier-Klutchko, Y. Eshdat, A. D. Strosberg, and L. J. Emorine. Human β_2-adrenergic receptors expressed in *Escherichia coli* membranes retain their pharmacological properties. *Proc. Natl. Acad. Sci. USA* **85**:7551–7555 (1988).
32. Granneman, J. G. Effects of agonist exposure on the coupling of β_1- and β_3-adrenergic receptors to adenylyl cyclase in isolated white adipocytes. *J. Pharmacol. Exp. Ther.* **261**:638–642 (1992).
33. Hausdorff, W. P., M. G. Caron, and R. J. Lefkowitz. Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB J.* **4**:2881–2889 (1990).
34. Arunlakshana, and H. O. Schild. Some quantitative uses of drug antagonists. *Br. J. Pharmacol.* **14**:48–58 (1959).
35. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).

Send reprint requests to: Kenneth P. Minneman, Ph.D., Department of Pharmacology, Emory University Medical School, Atlanta, GA 30322.